Human Molecular Genetics, 2008, Vol. 17, No. 8 **1175–1183**
doi:10.1093/hmg/ddn007
Advance Access published on January 8, 2008

Atypical Mowat–Wilson patient confirms the importance of the novel association between ZFHX1B/SIP1 and NuRD corepressor complex

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Received September 25, 2007; Revised and Accepted January 7, 2008

Mutations in *ZFHX1B* cause Mowat–Wilson syndrome (MWS) but the precise mechanisms underlying the aberrant functions of mutant ZFHX1B proteins (also named Smad-interacting protein-1, SIP1) in patients are unknown. Using mass spectrometry analysis, we identified subunits of the NuRD corepressor complex in affinity-purified Zfhx1b complexes. We find that Zfhx1b associates with NuRD through its N-terminal domain, which contains a previously postulated NuRD interacting motif. Interestingly, this motif is substituted by an unrelated sequence in a recently described MWS patient. We show here that such aberrant ZFHX1B protein is unable to recruit NuRD subunits and displays reduced transcriptional repression activity on the *X*BMP4 gene promoter, a target of Zfhx1b. We further demonstrate that the NuRD component Mi-2β is involved in repression of the Zfhx1b target gene *E-cadherin* as well as in Zfhx1b-induced neural induction in animal caps from *Xenopus* embryos. Thus, NuRD and Zfhx1b functionally interact, and defective NuRD recruitment by mutant human ZFHX1B can be a MWS-causing mechanism. This is the first study providing mechanistic insight into the aberrant function of a single domain of the multi-domain protein ZFHX1B/SIP1 in human disease.

INTRODUCTION

Loss-of-heterozygosity of *ZFHX1B* (encoding ZFHX1B, Zinc finger homeobox 1B, also named SIP1 and ZEB2) is implicated in the etiology of Mowat–Wilson syndrome (MWS). All patients with typical MWS present severe mental retardation and display distinctive facial dysmorphism in addition to multiple variable features, including heart defects, agenesis of the corpus callosum, seizures, urogenital anomalies and Hirschsprung disease (1). Analysis of the phenotype of *Zfhx1b* conventional knockout mouse embryos revealed defects in the neural crest cell lineage (2,3). In addition, the reduced expression of the neuroectodermal gene *Sox2* and the sustained expression of *E-cadherin* in the Zfhx1b defective neural plate indicated defects in early neurogenesis in these embryos (2). Both classes of defect are relevant to the neurological problems

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seen in MWS patients. Zfhx1b was also found to be a crucial regulator of early neural tissue formation in *Xenopus* (4).

We have identified Zfhx1b as a protein that binds to TGFβ family receptor activated Smad proteins and acts as a DNA-binding transcriptional repressor (5,6). After δEF1 (Zfhx1a), Zfhx1b became the second and only other member of the Zfhx1 family in vertebrates. These multi-domain repressors are characterized by the presence of a homeodomain-like domain and two flanking C_2H_2-type zinc finger clusters. Both clusters are necessary for Zfhx1b to repress target genes by binding to spaced CACCT(G) sequences in their regulatory region (6). Though studies have focused mainly on Zfhx1b repressive function, transcriptional activation activity has been ascribed to Zfhx1b as well (7,8). Indeed, Zfhx1b interacts not only with the corepressor CtBP (C-terminal binding protein) (9,10), but full-length Zfhx1b also binds to the coactivators p300 and pCAF (p300/CBP associated factor) (11). Taken together, Zfhx1-type proteins are complex multi-domain proteins: not all of their functions may depend on interaction with Smad proteins, and association with different partners via different domains may provide selectivity for target gene repression or activation.

The vast majority of the clinically characterized human mutations are all gene deletions, nonsense or frame shift mutations affecting a single allele. In nearly all cases, this leads to drastic C-terminal truncation of ZFHX1B in humans, and possibly instability of the protein (12). Recently, also non-truncating mutations in *ZFHX1B* in patients presenting with a mild form of MWS have been identified, leading to the assumption of a possible correlation between more subtle mutations and a milder or atypical phenotype (13–16). Despite the emerging list of mapped truncating and non-truncating mutations in *ZFHX1B*, the precise molecular mechanism(s) underlying the clinical features of MWS patients carrying these mutations are still unknown. In a global approach to address Zfhx1b's mechanism(s) of action, we decided to purify Zfhx1b protein complexes and identify candidate Zfx1b direct binding partners or proteins in its complex by mass spectrometry (MS). Doing so, we found a novel association of Zfhx1b with multiple subunits of the nucleosome remodeling and histone deacetylation complex NuRD. NuRD is composed of at least eight subunits, including the ATPase Mi-2β, which confers the chromatin remodeling activity (17). NuRD has been purified from both mammalian and amphibian cells and is considered to play a key role in transcriptional repression (18–21). Results from our study allow us to propose defective NuRD recruitment by Zfhx1b as part of Zfhx1b's mechanism of action and—here—as an underlying cause of a form of MWS.

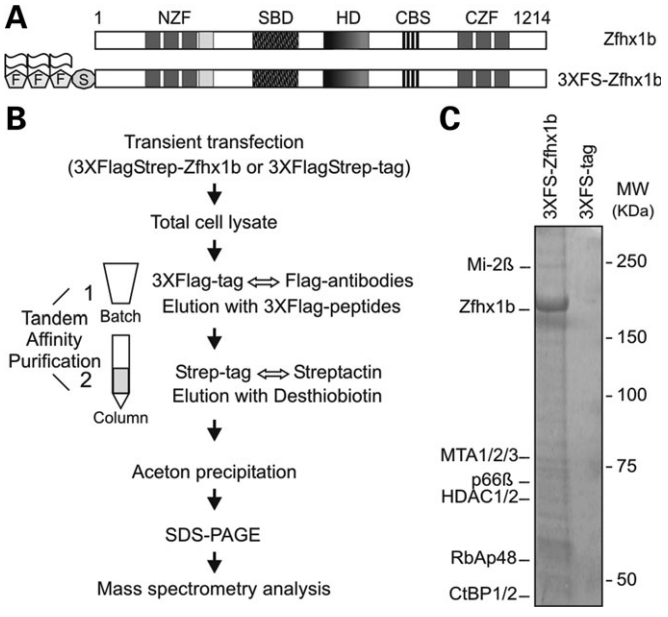

Figure 1. Tandem affinity purified Zfhx1b complexes contain multiple subunits of NuRD. (**A**) Graphic presentation of the mouse Zfhx1b protein and the N-terminally 3XFS-tagged variant. The N-terminal and C-terminal zinc finger domains (NZF and CZF), Smad-binding domain (SBD), homeodomain-like domain (HD), the C_3H type zinc finger (light grey box) and the CtBP interacting sites (CBS) are shown. (**B**) Schematic representation of the variant tandem affinity purification procedure. The first purification involves an in-batch Flag-IP of extracts of HEK293T cells, containing transiently synthesized 3XFS-Zfhx1b proteins. Upon elution with 3XFLAG peptides, the eluted proteins were applied to a streptactin-agarose column for a second purification. Precipitation was done using acetone. (**C**) Brilliant blue G-colloidal staining of the purified proteins from HEK293T cells transiently producing 3XFS-Zfhx1b or 3XFS-tag only. Co-purified proteins were identified by MS.

RESULTS

Affinity purified Zfhx1b complexes contain multiple NuRD subunits

We generated a tagged Zfhx1b protein in which a strep tag (S) is preceded by a 3XFlag epitope (F) at the N-terminus (Fig. 1A). Protein complexes containing this 3XFS-Zfhx1b were isolated by means of a variant tandem affinity purification approach (Fig. 1B). Briefly, total extracts of human embryonic kidney (HEK293T) cells transiently producing 3XFS-Zfhx1b proteins were subjected to anti-Flag agarose affinity gel-based purification. Following elution with excess 3XFlag peptide, the eluted proteins were applied to a streptactin agarose column as a second purification step. After gel electrophoresis and staining of the proteins, the bands were excised and the polypeptides were identified by MS (Fig. 1C). The major polypeptide, migrating as a protein of 170 kDa present in the purified 3XFS-Zfhx1b sample, was shown to correspond with 3XFS-Zfhx1b. Amongst the proteins that copurified with Zfhx1b were the known partners CtBP1 and -2 (9,10). Additionally, we identified novel Zfhx1b partners that are subunits of the NuRD complex, i.e. Mi-2β, p66β, the metastasis associated proteins MTA1, -2 and -3, the histone deacetylases HDAC1 and -2, and the retinoblastoma associated protein RbAp48.

Zfhx1b associates with NuRD

The identification of many subunits of NuRD in Zfhx1b complexes made us focus on a role of NuRD as a novel cofactor for Zfhx1b that is possibly involved in Zfhx1b-dependent repression. We therefore probed for Zfhx1b association with endogenous NuRD subunits. For this, we performed a Flag-based purification of extracts of HEK293T cells containing transiently produced 3XFS-Zfhx1b proteins (or only 3XFS

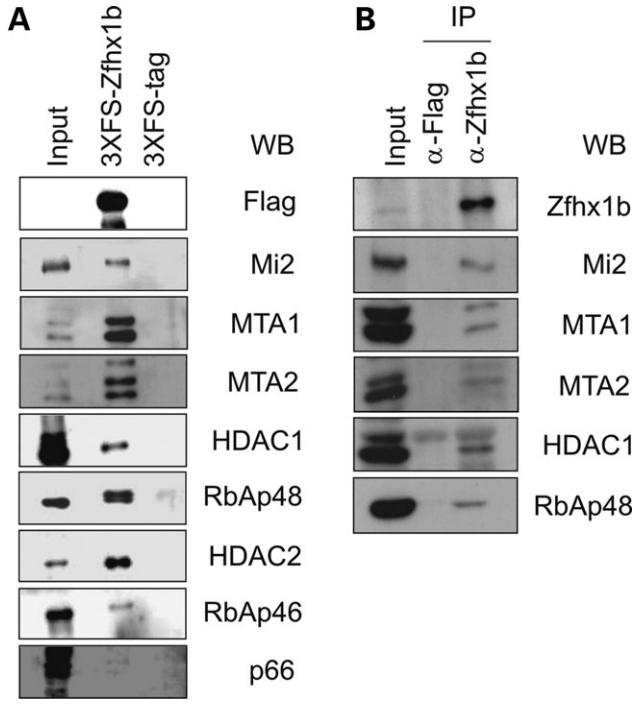

Figure 2. Zfhx1b associates with several subunits of the NuRD complex. (**A**) Extracts of HEK293T cells containing transiently synthesized 3XFS-Zfhx1b or 3XFS tag as negative control were precipitated in a Flag-dependent manner and eluted using 3XFLAG peptides. Western-blot analysis was performed using antibodies directed against the NuRD subunits. (**B**) IP of HEK293T cell extracts with Zfhx1b-specific or control anti-Flag antibodies, followed by Western-blot analysis using NuRD subunit-specific antibodies.

tag as control). Western-blot analysis using NuRD subunit-specific antibodies confirmed the association of Zfhx1b with all the subunits identified by MS, with the exception of p66β (Fig. 2A). Also RbAp46 of the NuRD complex co-purified with 3XFS-Zfhx1b. Subsequently, the interaction of endogenous Zfhx1b with endogenous NuRD subunits was analyzed by subjecting extracts of HEK293T cells to immunoprecipitation using anti-Zfhx1b antiserum. WB analysis showed that endogenous Zfhx1b associated with Mi-2β, MTA1, MTA2, HDAC1 and RbAp48 (Fig. 2B). None of the NuRD subunits were detected when extracts were incubated with control antibody. Hence, our results show that Zfhx1b associates with the NuRD complex at normal physiological levels. Similarly, we have also observed interaction between δEF1 and several NuRD subunits (data not shown).

Mi-2β is a specific cofactor for Zfhx1b-mediated transcriptional repression of E-cadherin promoter

Zfhx1b represses *E-cadherin* transcription in epithelial cells in culture, and an inverse correlation is seen between Zfhx1b and E-cadherin mRNA (as well as their protein levels) in cells of epithelium derived tumors (22). We previously observed a persistence of E-cadherin mRNA and protein in the neuroepithelium of *Zfhx1b* knockout mouse embryos, which has been linked with the neurological problems seen in MWS patients (2). These observations, together with DNA-binding data *in vitro*, make the *E-cadherin* gene a direct target for Zfhx1 proteins (6,10). To address a potential role for NuRD in Zfhx1b-regulated *E-cadherin* transcription, we included a mutant NuRD component, Mi-2βK750C, in an *E-cadherin* promoter-based reporter assay. Mi-2βK750C contains a mutation within its ATPase domain and was shown recently to act in a dominant-negative manner with respect to Mi-2β's involvement in NAB2-mediated repression (29). We found that the level of repression by Zfhx1b of *E-cadherin* promoter-based reporter constructs was reduced in the presence of Mi-2βK750C, whereas Mi-2βK750C alone did not have a substantial effect on this reporter (Fig. 3A). Overexpression of wild-type Mi-2β restored Zfhx1b-mediated transcriptional repression of the reporter construct (Fig. 3A). 3TPlux, which is another Zfhx1b-sensitive reporter (6), was not affected in the presence of Mi-2βK750C (Fig. 3B). These results demonstrate that Mi-2β can function as a target gene-specific corepressor of Zfhx1b.

A mutant Zfhx1b protein causing atypical MWS is unable to interact with NuRD

To identify the domain of Zfhx1b necessary for association with NuRD, a series of Zfhx1b deletion mutants (from mouse, Fig. 4A) were transiently produced and tested for interaction with MTA2, HDAC2 and RbAp48. As shown in Figure 4B, all peptides containing at least the Zfhx1b N-terminal domain co-precipitated with these subunits, whereas a long Zfhx1b polypeptide (from amino acid 715 till 1214) did not. Hence, the first 289 amino acids of Zfhx1b were found to be sufficient for interaction with the NuRD subunits tested here. An N-terminally 12 amino acid-long motif was recently found to be necessary and sufficient for NuRD recruitment by the transcriptional repressor Sall1 (30). Most notably, the critical residues within this motif that, upon substitution, abolished NuRD binding and decreased transcriptional repression by Sall1, are fully conserved within the Zfhx1b N-terminal domain. This putative NuRD-binding motif is also strongly conserved in Zfhx1b orthologues, including in *H. sapiens and X. laevis* (Fig. 5A; bold type sequences), as well as in δEF1.

A *ZFHX1B* mutation disrupting a splice site was recently identified in a patient presenting a mild form of MWS. The resulting protein was found to be identical to ZFHX1B except for the first 24 amino acids, which in this patient were replaced by a heterologous sequence (16). Alignment of wild-type and mutant sequences shows that this atypical ZFHX1B protein (referred to as AZmut) lacks the NuRD interaction motif (Fig. 5A). To test this experimentally, we generated a construct encoding a similar ZFHX1B mutant protein (see Fig. 5B), and probed for interaction with endogenous NuRD subunits. To this end, HEK293T cells extracts containing either transiently produced 3XFS-ZFHX1B$_{WT}$ or 3XFS-ZFHX1B$_{AZmut}$ proteins were subjected to immunoprecipitation with anti-Flag antibodies. Western-blot analysis using NuRD subunit-specific antibodies revealed defective NuRD association by ZFHX1B$_{AZmut}$ proteins, whereas CtBP binding was unaffected (Fig. 5C). Immuofluorescence analysis showed that wild-type as well as mutant ZFHX1B proteins both localize to the nucleus of HeLa cells (data not shown). Together, these findings support the importance of the

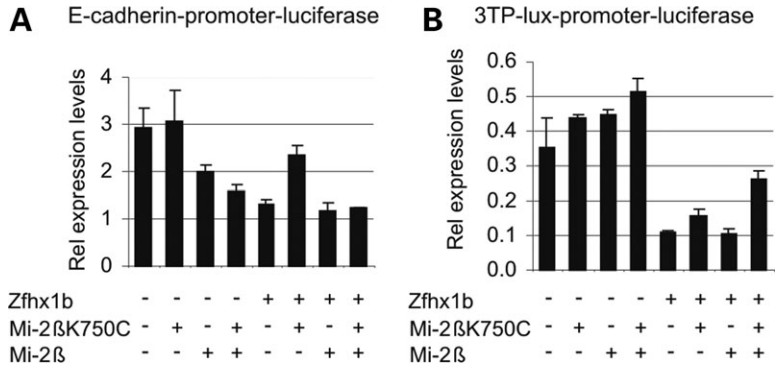

Figure 3. A dominant-negative variant of Mi-2β interferes with Zfhx1b-mediated repression of specific target promoters in reporter constructs. HEK293T cells were transiently transfected with constructs coding for Zfhx1b, wild-type or mutant Mi-2βK750C polypeptides, and the E-cadherin (**A**), or 3TPlux reporter constructs (**B**). Cells were harvested 48 h after transfection and were analyzed for luciferase activity. β-galactosidase activity corrected luciferase values are given.

conserved motif for NuRD recruitment by Zfhx1b protein, and further suggest defective NuRD recruitment by mutant ZFHX1B human protein as the underlying cause of this atypical MWS in this patient (16).

*X*Mi-2β is involved in *X*Zfhx1b-induced neural tissue formation

Zfhx1b plays an essential role in early neural development in the amphibian embryo (4). We therefore also used *Xenopus* as a model to investigate a potential role for NuRD in *X*Zfhx1b's neural inducing activity. First, we addressed whether the expression domain of endogenous *X*Mi-2β overlaps with that of *X*Zfhx1b during *Xenopus* embryogenesis. *In situ* hybridization analysis showed that *X*Mi-2β mRNA, like *X*Zfhx1b, is initially broadly expressed at gastrula stage, whereas at neurula stage it becomes more strongly expressed in neural tissue and neural crest cells. At tadpole stage, the strong expression of *X*Mi-2β and *X*Zfhx1b mRNA persists in the neural tube and also in neural crest cells and, also similar to mouse Zfhx1b mRNA, in the somites, in line with its documented role in somitogenesis (Fig. 6A) (31). This specific expression pattern thus supports a role for *X*Mi-2β in early neurogenesis, while the strong overlap between *X*Zfhx1b and *X*Mi-2β RNA expression suggests that the encoded proteins may indeed act together in neural development and possibly somitogenesis.

Part of *X*Zfhx1b's neuralizing activity can be ascribed to the transcriptional repression of *BMP4* and BMP-dependent genes (4,25,32). We therefore addressed also the ability of $ZFHX1B_{AZmut}$ proteins to repress expression driven from *X*BMP4 promoter-based reporter constructs in HEK293T cells. While wild-type ZFHX1B proteins repressed *X*BMP4 reporter constructs in a concentration-dependent manner, the repression activity of $ZFHX1B_{AZmut}$ proteins on this reporter construct was significantly reduced (Fig. 6B).To further investigate a role for NuRD in *X*Zfhx1b-neuralizing activity, we used a loss-of-function approach involving previously used *X*Mi-2β anti-sense MOs (28). Both the *X*Zfhx1b-dependent repression of *BMP4* and the BMP-dependent gene *XVent-1*, and the Zfhx1b-mediated induction of the neural marker *N-CAM* were reduced in animal cap explants derived from *X*Mi-2β MOs-injected *Xenopus* embryos (Fig. 6C). Co-injection of standard control MOs did not reduce *X*Zfhx1b activities to a similar extent. We therefore conclude that endogenous *X*Mi-2β is required for *X*Zfhx1b neuralizing activity. Co-injection of *h*Mi-2β RNA failed to rescue *X*Zfhx1b activity in the animal caps derived from *X*Mi-2β MOs injected embryos (Fig. 6B). Most likely, this is due to the mesoderm inducing activity seen upon Mi-2β overexpression, as described for *X*Mi-2 β recently (28).

DISCUSSION

Although the defects in neural crest and neurogenesis in conventional *Zfhx1b* knockout mice helped to explain the developmental defects that relate to certain clinical features of MWS patients, the precise molecular mechanism(s) underlying this congenital disorder is still unknown. Here, we followed a proteomics approach and identified multiple subunits of the NuRD complex as novel nuclear partners for Zfhx1b. We confirmed association of endogenous Zfhx1b with endogenous NuRD, and demonstrated that this interaction is mediated via the N-terminal domain of Zfhx1b, which harbors a recently defined NuRD-interacting motif. NuRD association has been reported for multiple transcriptional repressors, including Ikaros (33), KAP-1 (34), p53 (35), Bcl-6 (36), FOG-1 (37) and BCL11B (38). Previous purifications of the NuRD complex did not identify Zfhx1b proteins (18–21). This suggests that Zfhx1b is not a *bona fide* component of this complex but rather transiently associates, possibly depending on the cell type or its activation status, with NuRD to regulate transcription of a selective set of Zfhx1b target genes. In addition, previous searches for proteins containing the proposed NuRD-interaction motif yielded neither Zfhx1b nor δEF1 (Zfhx1a) (30,37). The reason may be 2-fold. First, all C_2H_2 type zinc finger transcriptional repressors that also interact with NuRD, such as Friend of GATA proteins (FOG-1 and FOG-2), Chicken Ovalbumin Upstream Promoter Transcription Factor Interacting Proteins (CTIP1 and CTIP2) and zinc finger protein 423 (Ebfaz), contain the motif at their extreme N-terminal end, whereas in Zfhx1b 11 amino acids unrelated to the proposed motif precede it. Second, the motif is in fact not strictly conserved within

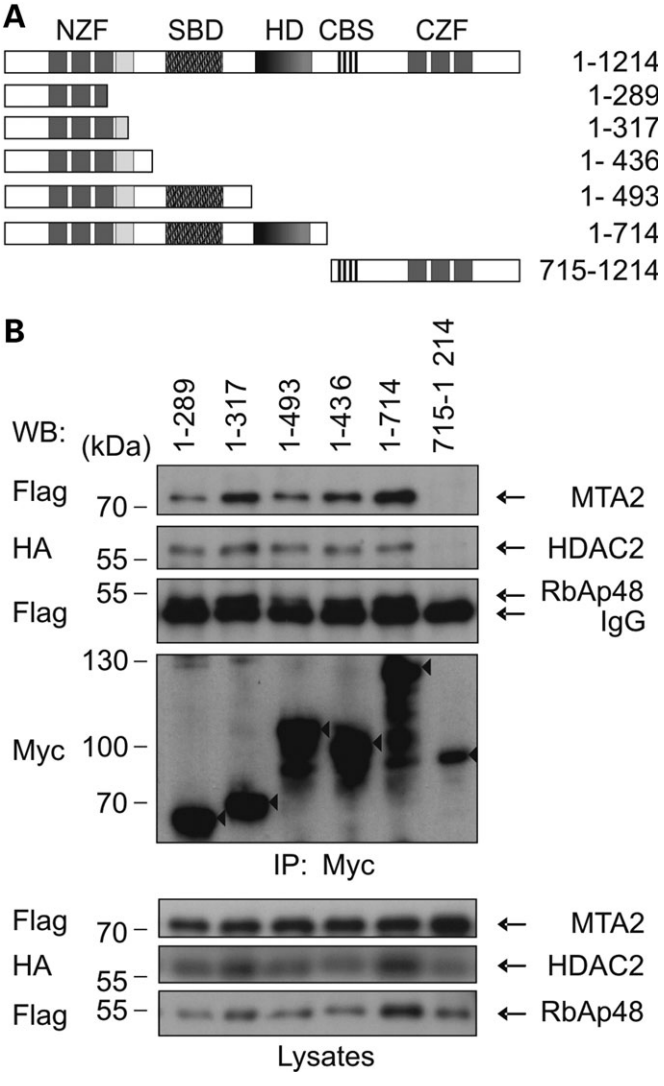

Figure 4. Zfhx1b associates with NuRD via its N-terminal domain. (**A**) Graphic presentation of wild-type (WT) full-length (FL) and mutant Zfhx1b proteins used in this study. (**B**) Western-blot detection showing co-immunoprecipitation of Flag-MTA2 and RbAp48, and HA-HDAC2 with the respective Zfhx1b proteins (triangle) tested.

Zfhx1b but still contains the critical residues found within Sall1 (30).

Patients presenting with typical MWS all carry either whole gene deletions or a translocation, nonsense mutation or a frame shift mutation in one allele (12). The clear lack of a correlation between the genotype and the clinical features of the patients previously suggested *ZFHX1B* haplo-insufficiency as the major mechanism causing MWS (39). Here, we present evidence suggesting that defective NuRD recruitment by ZFHX1B is implicated in the etiology of at least a mild form of MWS. First, a ZFHX1B protein, similar to the aberrant ZFHX1B protein in a patient presenting with atypical MWS (16), is unable to interact with NuRD. This aberrant protein in this patient is identical to the wild-type protein from amino acid 25 onwards, and therefore retains both zinc finger clusters required for DNA-binding and all previously

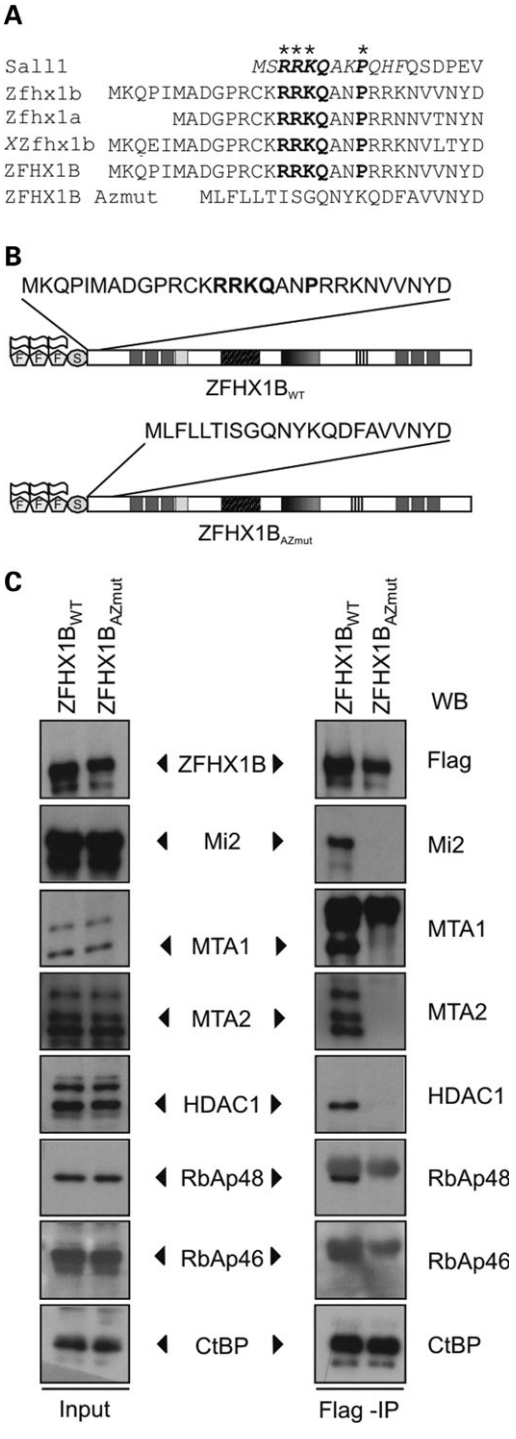

Figure 5. Defective NuRD recruitment by a mutant ZFHX1B protein that causes atypical MWS. (**A**) Alignment of Sall1, Zfhx1a, mouse, *Xenopus* laevis and human wild-type Zfhx1b, and mutant ZFHX1B amino-terminal domains. The conserved 12 amino acid motif (italics) in Sall1 that is sufficient for NuRD recruitment and the critical residues (*) that abolished binding to NuRD and repression by Sall1 when mutated are indicated. Note that mouse Zfhx1a and mouse, human and *Xenopus* laevis Zfhx1B but not the Atypical Zweier mutant human Zfhx1b contain the conserved motif (bold) within their N-terminus. (**B**) Schematic presentation of the ZFHX1B$_{WT}$ and ZFHX1B$_{AZmut}$ proteins used in this study. (**C**) Western-blot detection showing co-immunoprecipitation of endogenous NuRD subunits with ZFHX1B$_{WT}$ but not ZFHX1B$_{AZmut}$. Note that CtBP binding by ZFHX1B$_{AZmut}$ proteins is unaffected.

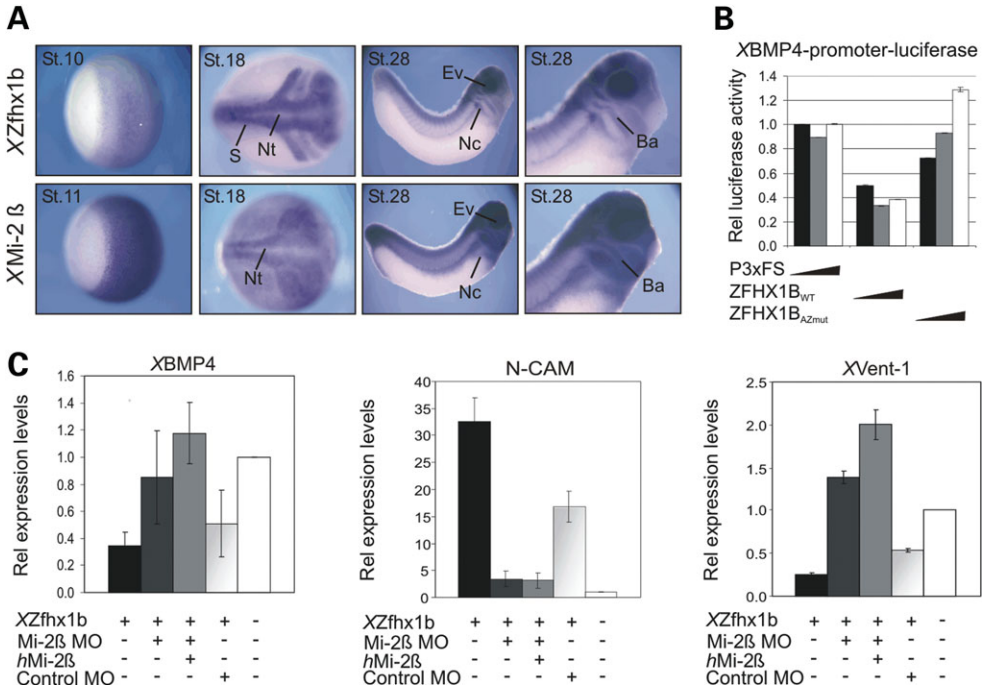

Figure 6. *X*Zfhx1b depends on intact *X*Mi-2β activity in early neural tissue formation. (**A**) Whole-mount *in situ* hybridization with a cRNA probe for *X*Zfhx1b and *X*Mi-2β at the indicated developmental stages; ba, branchial arches; ev, eye vesicle; nc, neural crest; nt, neural tube; s, somites. (**B**) The indicated plasmids were transfected into HEK293T cells and cells were analyzed 48 h later for the *X*BMP4 promoter-driven luciferase activity. β-Galactosidase-corrected luciferase values are given. Note that ZFHX1B$_{WT}$ represses the activity of the reporterconstruct in a dose-dependent manner, while repression by ZFHX1B$_{AZmut}$ proteins is significantly reduced. (**C**) QRT–PCR expression analysis of the indicated genes on total RNA isolated from animal caps derived from embryos injected with *X*Zfhx1b RNA alone (200 pg/blastomere), co-injected with MOs against *X*Mi-2β, co-injected with MOs against *X*Mi-2β and *h*Mi-2β RNA, co-injected with control MOs, or non-injected. Values were corrected for H4. Note that overexpression of *X*Zfhx1b represses *XBMP4* and the BMP-dependent gene *X*Vent-1 and induces the neural gene *N-CAM*, while knock-down of *X*Mi-2β alleviates *X*Zfhx1b-dependent repression and induction of the respective genes.

known interaction domains for non-NuRD proteins in Zfhx1b (16). Second, we found that the repression of *E-cadherin* promoter constructs by Zfhx1b is less efficient in the presence of a dominant-negative form of Mi-2β. Third, *X*Zfhx1b fails to efficiently induce a neural program in the absence of endogenous *X*Mi-2β in frog embryos, which might be relevant regarding the neurological problems seen in MWS patients. Fourth, we found that the ability of ZFHX1B$_{AZmut}$ proteins to repress expression driven from a *X*BMP4 promoter reporter construct is reduced compared to ZFHX1B$_{WT}$ proteins. Given the apparent specificity with which Zfhx1b and NuRD act together to selectively regulate promoters, this may very well explain the milder phenotype. We have shown previously that CtBP-mediated repression is only one of the mechanisms underlying Zfhx1b action, as observed in both Zfhx1b-induced neurogenesis in *Xenopus* (25) and *E-cadherin* repression (10). In line with this, multiple repression modes have also been reported for other transcriptional zinc finger repressors that recruit CtBP, including Knirps and FOG (40–42). The AZ patient still contains one functional *ZFHX1B* allele and the ZFHX1B$_{AZmut}$ protein misses only the first 24 amino acids. Hence, the resulting protein still contains all previously characterized domains, including a functional CtBP interacting domain (also shown in Fig. 5C). It is interesting to note that this patient has characteristic facial appearance but speaks full sentences. This indicates that the patient has also neural crest cell deficiencies, but the condition of mental retardation is quite different from that of typical MWS patients, who have severe mental retardation and limitation of language. Based on the presence of a potential N-myristoylation site within the highly evolutionary conserved N-terminal region affected in the AZmut patient, a critical role of ZFHX1B N-myristoylation in neural crest cell differentiation and migration was previously discussed (16). Our work, however, strongly supports a role for NuRD ZFHX1B association in neural crest cell development. Hence, the production of an aberrant ZFHX1B protein lacking the putative NuRD interacting motif within its N-terminal region but containing all previously characterized functional domains may well explain the clinical condition of the AZmut patient.

Given the evidence of NuRD-Zfhx1b association at physiological levels presented here, and the effect of *X*Mi-2β knockdown on *X*Zfhx1b activity in a process previously used to document Zfhx1b activity, our work strongly supports a role for the NuRD complex as a corepressor for *X*Zfhx1b activity, at least during neural tissue formation. Consistent with this, we previously found that this *X*Zfhx1b activity is sensitive to the HDAC inhibitor Trichostatin A (25). In addition to the very recently discovered role of Mi-2β in the positioning of the mesodermal/neurectodermal boundary by controlling on its turn *X*Zfhx1b transcripts (28), we show here a new aspect of Mi-2β as being a partner of Zfhx1b during early neural amphibian development. Taken together, our study provides new

molecular insight into *X*Zfhx1b neural inducing activity, which depends on its N-terminal domain and on interaction with NuRD. Finally, this study, which links a biochemical approach with mutation analysis in MWS patients, suggests that subtle mutations leading to milder or atypical MWS can be informative for the function of individual domains of the multi-domain Zfhx1-type proteins. Such more subtle mutations can in principle be introduced in mouse embryonic stem cells within the *Zfhx1b* gene itself for testing their effect on embryogenesis or postnatal development.

MATERIALS AND METHODS

Plasmids

The mouse 3XFS-Zfhx1b expression construct was generated by insertion of a strep tag encoding double-stranded oligonucleotide (AGCTTATGGCTAGCTGGAGCCACCCGCAGTT CGAAAAAGGCG) between the *Hin*dIII and *Eco*RI site of p3XFLAG-CMV-7.1 (Sigma), and subcloning of the full open reading frame of mouse Zfhx1b in frame with the N-terminally 3XFS tag (using *Bgl*II and *Eco*RV restriction sites). Constructs encoding Zfhx1b mutants were generated by subcloning the Zfhx1b cDNA fragments (eventually corresponding with amino acids 1–289, 1–317, 1–436, 1–493, 1–714 and 715–1214) in-frame with six Myc tags and the nuclear localization signal of SV40 T-antigen in pCS2-NLS-Myc. The cDNA encoding human ZFHX1B was first subcloned into pPCR-Script and via *Eco*RV and *Sma*I restriction digestion inserted into p3XFS (cut with *Eco*RV). The $ZFHX1B_{AZmut}$ was generated by insertion of selected double-stranded oligonucleotides containing the sequences as described (16). pCDNA3.1-FLAG-MTA2 and -RbAp48 were generated by insertion of the MTA2 and RbAp48 coding regions (pCMV Sport6 MTA2/RbAp48; Open Biosystems) into pCDNA3.1-FLAG. pMT2-HA-HDAC2 was received from S. Schreiber[0]. Constructs used in luciferase assays are the pGL3 wild-type E-cadherin promoter(−178+44)-luciferase reporter (22) and the p3TPLux reporter (23). From the 2 Kb-long *X*BMP4 (−2000 to +54) promoter-based luciferase reporter (received from W. Knochel), a 488 bp-long promoter fragment was amplified and cloned into pGL4.16. A cytomegalovirus promoter-driven *lacZ* reporter was used for normalization. pCMVSport6 with inserts of wild-type mouse Mi-2β and mutant *mouse* Mi-2βK750C cDNA were a kind gift from J. Svaren (Wisconsin-Madison; USA). The pCMV Sport6 hMi-2β was obtained from Open Biosystems. Constructs used in this study that have been described elsewhere are pCS2 with inserts of 6Myc-tagged wild-type mouse (5) and *Xenopus* Zfhx1b cDNA (24).

Cell culture and transient transfections

HEK293T cells were cultured in DMEM (Invitrogen) with 10% fetal bovine serum. Transient transfections for association studies were done with 25 kDa branched polyethyleneimine (PEI; Aldrich), using 4 μl of 1 mg of PEI/ml of stock solution per μg of DNA, unless stated elsewhere. Reporter assays were done according to van Grunsven *et al*. (25) using 7.5×10^4 cells per well of a 96-well plate.

Immunoprecipitations

For double affinity purification, HEK293T cells were seeded in 9 cm cell culture dishes (2×10^6 cells/dish) and transiently transfected after 24 h with 3XFS-tag or 3XFS-Zfhx1b-encoding plasmids. Cells were harvested 1 day later, washed twice with ice-cold phosphate-buffered saline and incubated for 20 min on ice with lysis buffer (170 mM NaCl, 10 mM EDTA, 50 mM Tris pH 7.4, 50 mM NaF, 0.2 mM dithiothreitol and 0.5% NP-40) supplemented with protease and phosphatase inhibitors. Lysates were collected by centrifugation (21.000 g for 20 min at 4°C). Supernatant was incubated with avidin (Biochemika) (4°C, 1 h) and avidin complexes were collected by centrifugation (20 min, 4°C). Extracts were subjected to affinity purification by overnight incubation with M2 anti-FLAG agarose beads (Sigma) on a rotating wheel at 4°C. Beads were washed and complexes were eluted by competition with 3XFLAG peptides (Sigma) (3 h, 4°C). Extracts were applied to a streptactin-agarose column for a second purification (carried out according to the manufacturer's protocol; IBA), and precipitated by incubation with two volumes of ice-cold acetone at −20°C. Semi-endogenous immunoprecipitation (IP) was done according to the conditions described for the tandem affinity purification, except for the avidin incubation, which was omitted here. Flag-affinity purified complexes were precipitated using acetone.

Co-IP. Whole cell extracts were precleared for 30 min using Protein G-Sepharose beads (Amersham Biosciences) and precipitated overnight at 4°C on a rotating wheel using antibodies against Myc (Santa Cruz) or Flag (Santa Cruz). Complexes were precipitated with blocked protein G Sepharose beads. For endogenous IP, HEK293T cells were plated in T-175 flasks and precipitation was carried out as described for co-IP. Precleared lysates were precipitated with anti-Zfhx1b rabbit polyclonal antiserum (10) and anti-Flag (Santa Cruz) antibodies. Antibodies used in western blotting detected CtBP (Sigma), Flag (Sigma), HDAC1 (Santa Cruz), HDAC2 (Santa Cruz), Mi2 (Santa Cruz), MTA-1 (Santa Cruz), MTA-2 (Santa Cruz), p66 (Upstate), RbAp46 (Santa Cruz), RbAp48 (Abcam) and Zfhx1b (10), respectively.

Mass spectrometry analysis

Purified Zfhx1b complexes were denatured for 5 min and subjected to gel electrophoresis. Staining of the gel was done with Brilliant Blue G-colloidal concentrate (Sigma), and bands were excised and subjected to MS (26). A nano-scale C18 reversed phase peptide separation system, on-line connected with the ion source of a Q-TOF1 mass spectrometer, was used (Micromass UK Cheshire, UK). Peptides were identified from their fragmentation spectra using the MASCOT search engine and the Swiss-Prot database. Peptides identified by a MASCOT score that exceeded the identity threshold score of MASCOT at the 95% confidence level were considered as positively identified.

Xenopus manipulations and injections

Embryos were obtained from adult frogs by hormone-induced egg-laying and *in vitro* fertilization using standard methods

(27). Capped transcripts were synthesized *in vitro* using the mMessage mMachine kit (Ambion, Austin, TX, USA) and injected in the animal hemisphere of all blastomeres of four cell stage embryos. Animal cap explants were prepared at late blastula stage (stage 9.5) and cultured in 1× Steinberg medium supplemented with 0.1% bovine serum albumin (Steinberg) until early neurula stage. The *X*Mi-2β and standard control morpholino oligonucleotides (MOs) were obtained from Gene Tools LLC and are described elsewhere (28).

In situ hybridization

Embryos were fixed in MEMFA, stained for β-galactosidase activity with 5-bromo 4-chloro-3-indolyl-β-galactopyranoside (X-Gal) and processed for *in situ* hybridization using digoxygenin-labeled antisense RNA probes (27). Production of the probe for detection of *X*Zfhx1b transcripts has been described elsewhere (24).

Real-time RT–PCR

The Qiagen RNeasy mini kit was used for RNA isolation from animal caps at stage 10. All RNA preparations were treated with DNAse I (Qiagen). Real-time RT–PCR was performed on an ABI PRISM 7700 (Applied Biosystems) using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). The following primers were used: BMP4 (F) 5′-GAGATTGTCCATT TCCCTTGGC-3′, (R) 5′-TCAGTGGAAAGAAGTCCAGC CG-3′; Histone H4, (F) 5′-CAAGCGCATCTCAGGTCTC AT-3′, (R) 5′-ACCTTGAGAACGCCACGAGT-3′; N-CAM (43); *X*Vent1, (F) 5′-CGATCCAACTCTACAGCCCC-3′, (R) 5′-GGGACTCAGAGATACGGAGGC-3′.

FUNDING

Fund of Scientific Research-Flanders (G.0279.04, G.0288.07, to D.H.); the interuniversity networks of the Federal Prime Minister (IUAP-P5/35 to D.H. and E.B., IUAP-P6/20 to D.H.).

ACKNOWLEDGEMENTS

We thank Magda Puype for expert technical assistance and Luc Nelles for valuable discussions. We also thank J. Svaren and W. Knochel for plasmids pCMVSport6 with inserts of wild type mouse Mi-2β and mutant mouse Mi-2βK750C cDNA, and the BMP4 promoter, respectively. G.V. was holder of a IWT predoctoral fellowship and was supported by a grant from the E. Vanderschueren Foundation, awarded by the Flanders League against Cancer. L.A.v.G. was holder of a post-doc position from the Fund of Scientific Research-Flanders and was also supported by a Krediet aan Navorsers project from the same agency. J.S. was holder of a post-doc position awarded by the Fonds de la Recherche Scientifique.

Conflict of Interest statement. The authors declare that they have no financial conflict of interests.

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